

PATENT COOPERATION T ATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
 US Department of Commerce
 United States Patent and Trademark
 Office, PCT
 2011 South Clark Place Room
 CP2/5C24
 Arlington, VA 22202
 ETATS-UNIS D'AMERIQUE
 in its capacity as elected Office

| | |
|---|---|
| Date of mailing (day/month/year) 26 April 2001 (26.04.01) | |
| International application No. PCT/IL00/00038 | Applicant's or agent's file reference 34/51 |
| International filing date (day/month/year) 20 January 2000 (20.01.00) | Priority date (day/month/year) 22 January 1999 (22.01.99) |
| Applicant GRESSEL, Jonathan et al | |

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
 17 August 2000 (17.08.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

| | |
|--|---|
| The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35 | Authorized officer Claudio Borton Telephone No.: (41-22) 338.83.38 |
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PATENT COOPERATION TREATY

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NOTIFICATION CONCERNING
AMENDMENTS OF THE CLAIMS(PCT Rule 62 and
Administrative Instructions, Section 417)

From the INTERNATIONAL BUREAU

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
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Date of mailing (day/month/year)

26 April 2001 (26.04.01)

International application No.

PCT/IL00/00038

International filing date (day/month/year)

20 January 2000 (20.01.00)

Applicant

YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al

The International Bureau hereby informs the International Preliminary Examining Authority that no amendments under Article 19 have been received by the International Bureau (Administrative Instructions, Section 417).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer

Claudio Borton

Facsimile No. (41-22) 740.14.35

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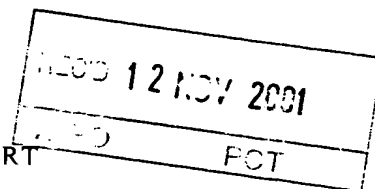
14

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)



| | | |
|--|--|---|
| Applicant's or agent's file reference 00/20549 | FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) | |
| International application No. PCT/IL00/00038 | International filing date (day/month/year) 20 JANUARY 2000 | Priority date (day/month/year) 22 JANUARY 1999 |
| International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet. | | |
| Applicant YEDA RESEARCH AND DEVELOPMENT CO., LTD. | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability: citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

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| Date of submission of the demand 17 AUGUST 2000 | Date of completion of this report 14 SEPTEMBER 2001 |
| Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 | Authorized officer <i>Medina A. Ibrahim</i> MEDINA A. IBRAHIM |
| Facsimile No. (703) 305-3230 | Telephone No. (703) 308-0196 |

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/IL00/00038

I. Basis of the report

1. With regard to the **elements** of the international application:*☒ the international application as originally filed☒ the description:pages 1-26, as originally filedpages NONE, filed with the demandpages NONE, filed with the letter of _____☒ the claims:pages 27-38, as originally filedpages NONE, as amended (together with any statement) under Article 19pages NONE, filed with the demandpages NONE, filed with the letter of _____☒ the drawings:pages 1-2, as originally filedpages NONE, filed with the demandpages NONE, filed with the letter of _____☒ the sequence listing part of the description:pages 1-5, as originally filedpages NONE, filed with the demandpages NONE, filed with the letter of _____2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).☐ the language of publication of the international application (under Rule 48.3(b)).☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:☒ contained in the international application in printed form.☒ filed together with the international application in computer readable form.☐ furnished subsequently to this Authority in written form.☐ furnished subsequently to this Authority in computer readable form.☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.4. ☒ The amendments have resulted in the cancellation of:☒ the description, pages NONE☒ the claims, Nos. NONE☒ the drawings, sheets/fig. NONE5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

**Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

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III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 8-12, 19-21, 29-31, 67-69, 77-79

because:

- ☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination (*specify*).

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. _ are so unclear that no meaningful opinion could be formed (*specify*).

- ☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

- ☒ no international search report has been established for said claims Nos. (See Attached).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/IL00/00038

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. statement**

Novelty (N)

| | | |
|--------|--|-----|
| Claims | <u>(Please See supplemental sheet)</u> | YES |
| Claims | <u>(Please See supplemental sheet)</u> | NO |

Inventive Step (IS)

| | | |
|--------|--|-----|
| Claims | <u>(Please See supplemental sheet)</u> | YES |
| Claims | <u>(Please See supplemental sheet)</u> | NO |

Industrial Applicability (IA)

| | | |
|--------|--|-----|
| Claims | <u>(Please See supplemental sheet)</u> | YES |
| Claims | <u>(Please See supplemental sheet)</u> | NO |

2. citations and explanations (Rule 70.7)

Claims 1-7, 13-18, 22-28, 32-33, 62-66, 70-76, 80-83 lack an inventive step under PCT Article 33(3) as being obvious over Brugliera et al (US 5,859,334) in view of Bar-Peled et al, and further in view of Lewinsohn et al.

Claims are drawn to an isolated polynucleotide comprising a nucleotide sequence, from a citrus plant, encoding a polypeptide having a flavanone-7-O-glucoside-2"-O-rhamnsyltransferase expressed in a plant cell either in the antisense orientation or in co-suppression to reduce production of flavanone-7-O-glucoside-2"-O-rhamnsyltransferase in said plant or plant cell.

Brugliera et al teach isolated sequences encoding UDP-rhamnose: anthocyanidin-3-glucoside rhamnosyltransferase (3RT)(see, columns 8,15, 19-20), and transgenic plant or plant cells expressing said sequence either in sense or antisense orientation for an altered phenotype(see, columns 10-11, 23-24). Brugliera et al does not expression of flavanone-7-O-glucoside-2"-O-rhamnsyltransferase in transgenic plants.

However, Bar-Peled et al teach isolated and purified flavanone-7-O-glucoside-2"-O-rhamnsyltransferase , and suggest the isolation of its gene for use in genetic engineering to modify bitterness in grapefruit (see, page 20953, Abstract; pages 20954, and last paragraph of page 20956).

Lewinsohn et al teach the importance of flavanone-7-O-glucoside-2"-O-rhamnsyltransferase from citrus plants.

Therefore it would have obvious to one of ordinary skill in the art to use the method of transforming a plant or plant cell with an isolated rhamnosyltransferase gene as taught by Brugliera et al, and to modify that method by incorporating the flavanone-7-O-glucoside-2"-O-rhamnsyltransferase gene suggested by Bar-Peled et al, given the teaching by Lewinsohn of importance of the citrus flavanone-7-O-glucoside-2"-O-(Continued on Supplemental Sheet.)

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/IL00/00038

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): C12N 5/14, 15/09, 15/11, 15/29, 15/54, 15/63, 15/82; A01H 5/00

US CL: 800/278, 286, 295, 316; 536/23.1, 23.2, 23.6, 24.5; 435/69.1

III. NON-ESTABLISHMENT OF REPORT:

No international search report has been established for claim numbers 8-12, 19-21, 29-31, 67-69, 77-79.

V. 1. REASONED STATEMENTS:

The report as to Novelty was positive (YES) with respect to claims 1-7, 13-18, 22-28, 32-33, 62-66, 70-76, 80-83.

The report as to Novelty was negative (NO) with respect to claims NONE.

The report as to Inventive Step was positive (YES) with respect to claims NONE.

The report as to Inventive Step was negative (NO) with respect to claims 1-7, 13-18, 22-28, 32-33, 62-66, 70-76, 80-83.

The report as to Industrial Applicability was positive (YES) with respect to claims 1-7, 13-18, 22-28, 32-33, 62-66, 70-76, 80-83.

The report as to Industrial Applicability was negative (NO) with respect to claims NONE.

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

rhamnsyltransferase, to produce transgenic plants or plant cell with modified phenotype as taught by Brugliera et al. with a reasonable expectation of success.

Claims 1-7, 13-18, 22-28, 32-33, 62-66, 80-83 meet the criteria set out in PCT Article 33(2), because the prior art does not teach the claimed isolated polynucleotide comprising a nucleotide sequence from a citrus plant encoding a polypeptide having a flavanone-7-O-glucoside-2"-O-rhamnsyltransferase or transgenic plants or plant cells expressing it .

Claims 1-7, 13-18, 22-28, 32-33, 62-66, 80-83 meet the criteria set out in PCT Article 33(4), because production of transgenic plants or plant cells with reduced bitterness have importance in agricultural economics.

----- NEW CITATIONS -----

NONE

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Statement under Article 35(2) with regard to novelty, inventive step or industrial applicability;
 Citations and explanations supporting such statement

1. statement

| | | | |
|-------------------------------|--------|---------------------------------|-----|
| Novelty (N) | Claims | (Please See supplemental sheet) | YES |
| | Claims | (Please See supplemental sheet) | NO |
| Inventive Step (IS) | Claims | (Please See supplemental sheet) | YES |
| | Claims | (Please See supplemental sheet) | NO |
| Industrial Applicability (IA) | Claims | (Please See supplemental sheet) | YES |
| | Claims | (Please See supplemental sheet) | NO |

2. citations and explanations (Rule 70.7)

Claims 1-7, 13-18, 22-28, 32-33, 62-66, 70-76, 80-83 lack an inventive step under PCT Article 33(3) as being obvious over Brugliera et al (US 5,859,334) in view of Bar-Peled et al, and further in view of Lewinsohn et al.

Claims are drawn to an isolated polynucleotide comprising a nucleotide sequence, from a citrus plant, encoding a polypeptide having a flavanone-7-O-glucoside-2"-O-rhamnosyltransferase expressed in a plant cell either in the antisense orientation or in co-suppression to reduce production of flavanone-7-O-glucoside-2"-O-rhamnosyltransferase in said plant or plant cell.

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Therefore it would have obvious to one of ordinary skill in the art to use the method of transforming a plant or plant cell with an isolated rhamnosyltransferase gene as taught by Brugliera et al, and to modify that method by incorporating the flavanone-7-O-glucoside-2"-O-rhamnosyltransferase gene suggested by Bar-Peled et al, given the teaching by Lewinsohn of importance of the citrus flavanone-7-O-glucoside-2"-O-(Continued on Supplemental Sheet.)

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (51) International Patent Classification ⁷ : C12N | A2 | (11) International Publication Number: WO 00/43490 (43) International Publication Date: 27 July 2000 (27.07.00) |
| (21) International Application Number: PCT/IL00/00038 (22) International Filing Date: 20 January 2000 (20.01.00) (30) Priority Data: 128193 22 January 1999 (22.01.99) IL (71) Applicants (for all designated States except US): YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL/IL]; Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL). STATE OF ISRAEL - MINISTRY OF AGRICULTURE [IL/IL]; The Volcani Center, P.O. Box 6, 50200 Bet Dagan (IL). (72) Inventors; and (75) Inventors/Applicants (for US only): GRESSEL, Jonathan [IL/IL]; Hayarden Street 15, 76604 Rehovot (IL). EYAL, Yoram [IL/IL]; Habrigada Street 21, 76228 Rehovot (IL). FLUHR, Robert [US/IL]; Ben-Yehuda Street 19/3, 76301 Rehovot (IL). (74) Agent: FRIEDMAN, Mark, M.; Beit Samueloff, Haomanim Street 7, 67897 Tel Aviv (IL). | | (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i> |
| (54) Title: RHAMNOSYL-TRANSFERASE GENE AND USES THEREOF | | |
| (57) Abstract An isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide having a flavanone- 7-O-glucoside-2"-O-rhamnosyl- transferase catalytic activity and its uses. | | |

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RHAMNOSYL-TRANSFERASE GENE AND USES THEREOF

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a nucleic acid encoding a 1-2-rhamnosyl-transferase and uses thereof. More particularly, the present invention relates to a multistep process of converting hesperidin from orange peels to the sweetener neohesperidin dihydrochalcone (NHDC), in which, in one of the steps, 1-2-rhamnosyl-transferase in the presence of activated rhamnose is used in a rhamnosylation reaction to convert hesperidinase-treated hesperidin (H7G) to neohesperidin. Further particularly, the present invention relates to genetically modified plants of the *Citrus* genus including an antisense or sense (for co-suppression) construct which comprises the above nucleic acid or a gene knock-out integrated construct to provide less bitter grapefruits, pomelos and other citrus containing bitter flavanoid glycosides.

The bitter flavanones naringin and neohesperidin (Figure 1) are produced only in young leaves and fruits of a few citrus species, such as grapefruit and pomelo, accumulate in a brief few week period, and remain through maturity (Castillo et al., 1992). Isomerically structured, yet tasteless flavanones, such as hesperidin (Figure 1) are produced in oranges at the same time in development (Castillo et al., 1993). The differences between the tastelessness of orange hesperidin and the bitterness of grapefruit and pomelo naringin and neohesperidin depend on a specific set of glycosylation reactions (glucosylation and rhamnosylation) as further detailed hereinunder.

Low consumption of grapefruit is due in part to the bitter flavor of its juice and flesh (Matthews et al., 1990). This bitterness is due to the presence of large amounts of the flavanone glycoside naringin, as well as limonin in the juice. While limonin is a problem limited to the juice (Guadagni et al., 1973), in the intact fruit it appears in the tasteless A ring monolactone and forms the bitter dilactone only after maceration in the acidic juice environment (Matthews et al., 1990).

Thus, bitterness in the fruit can be decreased by reducing naringin levels. The bitterness in commercially prepared grapefruit juice is presently diminished to levels more acceptable by consumers by using resins that adsorb some bitter compounds or by treating the juice with the immobilized enzyme naringinase (Jimeno et al., 1987; Nikdel et al., 1989).

Commercial naringinase preparations typically consist of two enzymes, mainly α -rhamnosidase and some β -glucosidase, which successively hydrolyze one or both the sugar groups from naringin, leaving the tasteless compound prunin (naringenin-7-*O*-glucoside). Removal of the terminal rhamnose removes about 95 % of the bitterness. Evidently, this procedure does not solve the problem of bitterness of whole fruit or home prepared juice.

A better approach which also addresses the above problem would, therefore, be to regulate the levels of naringin within the plant itself.

However, because of high heterozygosity of the commercial citrus varieties, classical plant breeding programs, will be hard put to yield identical varieties, yet having significantly less bitter fruit by reducing naringin. It will be appreciated in this respect that the presently available low naringin varieties (such as the Texas Red) are also low yielding.

While reducing the present invention to practice, studies of the naringin flavonoid metabolism (see below) assisted (i) in developing novel approaches to the problem; (ii) to further study the critical glycosylation steps that produce naringin; and (iii) in using this knowledge to modulate the degree of bitterness.

Citrus flavonoids ubiquity and biosynthesis: The genus *Citrus* contains many flavonoid glycosides that differ either in the structure of the aglycone or their sugar moieties. The major flavonoid in pomelo and grapefruit peel is naringin, while sweet orange peel contains hesperidin (Horowitz and Gentili, 1977 and Figure 1). Peels of sour orange, trifoliate orange and Ponderosa lemon contain neohesperidin (Figure 1). Neohesperidin and naringin are flavanone glycosides that contain the same disaccharide, β -neohesperidose (2-*O*- α -L-rhamnosyl- β -D-glucose), which is attached at position C-7 of different aglycones, hesperetin and naringenin, respectively. Hesperidin and narirutin are isomers, respectively, of the above compounds that contain the disaccharide rutinose (6-*O*- α -L-rhamnosyl- β -D-glucose).

These flavonoids have some remarkable taste properties. Naringin and neohesperidin are extremely bitter, while narirutin and hesperidin are nearly tasteless (Horowitz and Gentili, 1986; Naim et al., 1986).

Flavonoids in Citrus: The highest naringin levels are associated with very young developing leaves and fruit tissue. Undifferentiated cells of *Citrus paradisi* (grapefruit) were able to biotransform exogenous naringenin and hesperetin, into prunin (naringenin-7-*O*-glucoside) and

hesperetin-7-*O*-glucose (H-7-G), respectively. Further 1-2-rhamnosylation resulting in naringin or neohesperidin synthesis was not detected, although 1-6-rhamnosylation resulting in narirutin was observed (Lewinsohn et al., 1986, 1989b).

All of the above suggests that the blockage in the stepwise production of the naringenin from prunin (naringenin-7-*O*-glucoside) in the undifferentiated *Citrus* cells is caused by the absence of a specific rhamnosyl transferase activity.

Lewinsohn et al. detected chalcone-synthase and UDP-glucose, flavanone-7-*O*-glucosyl-transferase activities in cell-free extracts of *Citrus* (Lewinsohn et al. 1989a). Partial purification of the glucosyl transferase has been recently reported and some of its characteristics are therefore known (McIntosh and Mansell, 1989).

The glucosylated flavanone was further rhamnosylated. Chalcone-synthase activity was detected in cell-free extracts derived from young leaves and fruits.

Young fruits (2 millimeter diameter) had the highest chalcone synthase activity. In earlier studies it was shown that the glycosylation of the aglycone, naringenin, in undifferentiated *Citrus* cells occurs in two steps. First there was an initial glucosylation resulting in prunin (Lewinsohn et al. 1986, 1989b), and then a further rhamnosylation of prunin occurred when exogenous UDP-glucose and NADPH were added to the extract forming the end-product naringin (Lewinsohn et al 1989b).

Prunin was also shown to be a likely intermediate in the biosynthesis of naringin in immature grapefruit fruits (Berhow and Vandercook 1989).

Several glucosyl-transferases from plants catalyze the transfer of the sugar moiety from an activated UDP-sugar to a specific site on the flavonoids (Hahlbrock, 1981). Enzymatic preparations from plants catalyze the conversion of UDP-glucose to UDP-rhamnose in the presence of NADPH (Barber, 1962). The conversion is due to at least three different enzymatic activities requiring NADH or NADPH. It was not previously known how this conversion is catalyzed in *Citrus*. A coupled assay using UDP-glucose with NADPH was developed, which formed a rhamnosylated flavanone glycoside, indicative of a UDP-rhamnose:flavanone-7-*O*-glucoside-2'-*O*-rhamnosyl-transferase activity (α -1-2 rhamnosyl transferase). A system was thereafter developed to directly biosynthesize radiolabeled UDP-rhamnose from [^{14}C]- UDP glucose and NADPH for use in direct assay of the α -1-2 rhamnosyl transferase activity during

purification of the α -1-2 rhamnosyl transferase: The direct assay facilitated the purification of the α -1-2 rhamnosyl transferase. Once both rhamnosyl transferase substrates were available it was possible to purify the enzyme. Purity to homogeneity of the α -1-2 rhamnosyl transferase in a four step
5 procedure, which includes S-200 gel filtration, affinity column, ion exchange-FPLC and reverse-phase HPLC (Bar-Peled et al. 1991) was achieved and an antibody to it was elicited. These findings allowed to isolate the gene, which is the subject of this invention.

Semi-artificial sweetener: Neohesperidin and naringin from the
10 peels of sour oranges and grapefruits are being converted into neohesperidin dihydrochalcone (NHDC), an intense sweetener used in the EU that is 2,000 fold sweeter than sugar (see right path on Figure 1). NHDC is especially useful when long-lasting sweetness is desired, such as in chewing gum or in admixture with one of the artificial sweeteners. Currently, world-wide
15 production of this NHDC is limited by the amount of neohesperidin and naringin available from these lesser grown *Citrus* species (less than 10 % of total *Citrus* cultivated).

Unfortunately, the vast majority of citrus processed (oranges) contain large amounts of the hesperidin, an isomer having the terminal rhamnose
20 attached by a 1 \rightarrow 6 linkage instead of the 1 \rightarrow 2 linkage. Hesperidin cannot be converted to the sweetener and is currently a waste by-product.

There is thus a widely recognized need for, and it would be highly advantageous to have, a nucleic acid encoding a 1-2-rhamnosyl-transferase to effect a multistep process of converting hesperidin from orange peels to
25 the sweetener neohesperidin dihydrochalcone (NHDC), and to provide genetically modified plants of the *Citrus* genus including an antisense or sense (for co-suppression) construct thereof, or knock-out integrated construct, to provide less bitter grapefruits, pomelos and other citrus containing bitter flavanoid glycosides.

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SUMMARY OF THE INVENTION

While reducing the present invention to practice, a novel rhamnosyl transferase gene from citrus responsible for producing the bitter flavanoids naringin and neohesperidin was isolated, sequenced, and characterized.
35 This gene can be used in antisense or overproducing sense constructs to decrease the bitterness in grapefruit, and in sense constructs as part of the bioconversion of hesperidin from orange waste products to neohesperidin, used for the production of neohesperidin dihydrochalcone (NHDC), a

sweetener. A knock-out construct can be used to knock-out the endo rhamnosyl transferase gene.

Thus, according to one aspect of the present invention there is provided an isolated polynucleotide comprising a nucleotide sequence
5 encoding a polypeptide having a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase catalytic activity.

According to another aspect of the present invention there is provided a cell genetically modified to include, in an expressible sense orientation, a nucleotide sequence encoding a flavanone-7-*O*-glucoside-2"-
10 *O*-rhamnosyl-transferase.

According to still another aspect of the present invention there is provided a plant cell of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, wherein the plant cell is genetically modified to include, in an expressible antisense
15 orientation, a nucleotide sequence encoding an antisense RNA molecule being capable of *in vivo* base pairing with the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, to thereby render the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, when expressed, amenable to degradation by nucleases present in the plant cell.

According to yet another aspect of the present invention there is provided a transgenic plant of a species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, wherein the transgenic plant is genetically modified to include, in an expressible antisense orientation, a nucleotide sequence encoding an antisense RNA
20 molecule being capable of *in vivo* base pairing with the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, to thereby render the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, when expressed, amenable to degradation by nucleases present in the transgenic plant.

According to a further aspect of the present invention there is provided a plant cell of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, wherein the plant cell is genetically modified to include, in an overexpressible sense orientation, a nucleotide sequence encoding a sense RNA molecule being capable of
25 inducing a co-suppression effect to thereby reduce production of the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the plant cell.

According to yet a further aspect of the present invention there is provided a transgenic plant of a species naturally expressing a flavanone-7-

O-glucoside-2"-*O*-rhamnosyl-transferase, the plant is genetically modified to include, in an overexpressible sense orientation, a nucleotide sequence encoding a sense RNA molecule being capable of inducing a co-suppression effect to thereby reduce production of the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the plant cell.

According to still a further aspect of the present invention there is provided a transgenic plant or cell of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, the transgenic plant or cell being genetically modified to knock-out a gene encoding said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase to thereby abolish production of said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the transgenic plant or cell.

According to an additional aspect of the present invention there is provided a commercial process of producing neohesperidin comprising the steps of: (a) extracting hesperidin from a hesperidin producing species of the genus *Citrus*; (b) treating the hesperidin with a hesperidinase, thereby obtaining hesperetin-7-glucoside; and (c) treating the hesperetin-7-glucoside with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose, thereby obtaining neohesperidin.

According to yet another additional aspect of the present invention there is provided a method of modifying a rhamnose-1-6-glucose linkage of a chemical compound to a rhamnose-1-2-glucose linkage comprising the steps of: (a) enzymatically treating the chemical compound to remove a rhamnose group thereof, thereby obtaining a first derivative of said chemical compound having a terminal glucose group; and (b) enzymatically treating the first derivative with recombinant flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose, thereby obtaining a second derivative chemical compound having the rhamnose-1-2-glucose linkage.

According to one further aspect of the present invention there is provided a commercial process of producing neohesperidin dihydrochalcone comprising the steps of: (a) extracting hesperidin from a hesperidin producing species of the genus *Citrus*; (b) treating the hesperidin with a hesperidinase, thereby obtaining hesperetin-7-glucoside; (c) treating the hesperetin-7-glucoside with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose, thereby obtaining neohesperidin; (d) treating the neohesperidin with an alkali, thereby

obtaining neohesperidin chalcone; and (e) reducing the neohesperidin chalcone, thereby obtaining neohesperidin dihydrochalcone.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a nucleic acid encoding a 1-2-rhamnosyl-transferase to effect a multistep process of converting hesperidin from orange peels to the sweetener neohesperidin dihydrochalcone (NHDC), and to provide genetically modified plants of the *Citrus* genus to provide less bitter grapefruits, pomelos and other citrus containing bitter flavanoid glycosides.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 depicts a prior art process (right path) and a process according to the present invention (left path) of producing the sweetener NHDC; and

FIG. 2 shows the sequence of the coding strand of a 1-2-rhamnosyl-transferase cDNA according to the present invention, and its translation into a protein shown in single letter code.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a 1-2-rhamnosyl-transferase gene and the recombinant protein product hereof, which can be used in a multistep process of converting hesperidin from orange peels to the sweetener neohesperidin dihydrochalcone (NHDC), and in providing genetically modified plants of the genus *Citrus* characterized by lowered bitterness.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting. Generally, the nomenclature used herein and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art.

While reducing the present invention to practice, a polynucleotide was isolated, having a nucleotide sequence encoding a polypeptide having a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase catalytic activity. The isolation of the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase gene was not at all trivial. Repeated attempts to isolate the gene via screening of either conventional cDNA libraries or expression cDNA libraries using oligonucleotides as well as antibodies raised against purified flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase failed to yield positive clones. The successful approach of isolating the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase gene, which included reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate primers followed by rapid amplification of cDNA ends (RACE), is further described in the Examples section that follows.

Having the cDNA in hand, screening of genomic libraries can readily be performed for isolating a genomic clone including a polynucleotide having introns and exons, the exons of which correspond to the cDNA sequence or parts thereof, and encode for flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase. Both the complementary and genomic polynucleotides according to the present invention can be used to direct the synthesis of a messenger RNA (mRNA) encoding flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase.

According to a preferred embodiment of the present invention, the isolated polynucleotide, either in its cDNA or genomic DNA form, wholly or partially synthetic (the term "isolated" may encompass all these possibilities) is ligated to a vector, preferably, an expression vector, either in sense or antisense orientation. The vector preferably selected is propagatable in plant cells and/or in microorganism cells, such as in bacteria. The vector can be of a type capable of directing the integration of sequences thereof into the genome of the recipient cell. Thus, within the cell, the nucleic acid may be incorporated, or not, within a chromosome. There may be more than one nucleotide sequence per haploid genome. This, for example, enables increased expression. A vector comprising a polynucleotide according to the present invention need not include a promoter, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

However, as further detailed hereinunder, a promoter is preferably included in the vector.

The present invention also encompasses the expression product of any of the nucleic acid sequences disclosed and methods of making the expression product by expression from encoding nucleic acid therefor under suitable conditions in suitable host cells. Those skilled in the art are well able to construct vectors and design protocols for expression and recovery of products of recombinant gene expression.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al. 1989, Cold Spring Harbor Laboratory Press. Transformation procedures depend on the host used, but are well known. Many known techniques and protocols for manipulation of nucleic acids, for example, in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. ed., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

According to one embodiment of the present invention the nucleotide sequence is as set forth in SEQ ID NO:20 or a functional part thereof. By functional part it is meant a part capable of directing the synthesis of a polypeptide having a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase catalytic activity.

According to another embodiment of the present invention the nucleotide sequence shares between 50 and 100 %, preferably between 60 and 100 %, more preferably between 70 and 100 %, yet more preferably between 80 and 100 %, still more preferably between 90 and 100 % identical bases with SEQ ID NO:20 or a functional part thereof, as determined using a sequence analysis software package developed by the Genetic Computer Group (GCG) at the University of Wisconsin with gap creation penalty of 50 and gap extension penalty of 3, provided that the nucleotide sequence encodes a polypeptide having a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase catalytic activity.

According to yet another preferred embodiment of the present invention, the nucleotide sequence is hybridizable with SEQ ID NO:20 or a functional part thereof under stringent hybridization, moderate hybridization or mild hybridization, wherein stringent hybridization is effected by a

hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, moderate hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C, whereas mild hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50 °C, final wash solution of 2 x SSC and final wash at 40 °C. Thus, a hybridization according to the present invention is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50-65 °C, final wash solution of 0.1-2.0 x SSC and final wash at 40-60 °C.

According to a preferred embodiment of the present invention, the nucleotide sequence encodes a polypeptide as set forth in SEQ ID NO:21 or a functional part thereof. However, the polypeptide encoded by the nucleotide sequence of the present invention can share between 20 and 100 %, preferably between 60 and 100 %, more preferably between 70 and 100 %, yet more preferably between 80 and 100 %, still more preferably between 90 and 100 % identical or conserved amino acids with SEQ ID NO:21 or a functional part thereof, as determined using a sequence analysis software package developed by the Genetic Computer Group (GCG) at the University of Wisconsin with gap creation penalty of 12 and gap extension penalty of 4, provided that the polypeptide has a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase catalytic activity.

A polynucleotide according to various aspects of the present invention may have the sequence of a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase gene or be a mutant, variant, derivative or allele of the sequence provided. Preferred mutants, variants, derivatives and alleles are those which encode a product (RNA or polypeptide) which retains a functional characteristic of the product encoded by the wild-type gene. Changes to a sequence, to produce a mutant, variant or derivative, may be by one or more of addition, insertion, deletion or substitution of one or more amino acids in an encoded polypeptide product. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included.

In a preferred embodiment of the present invention a polynucleotide molecule comprises a nucleotide sequence which encodes an amino acid sequence shown in SEQ ID NO:21. The nucleotide sequence may comprise an encoding sequence shown in SEQ ID NO:20 or may be a mutant, variant,

derivative or allele thereof encoding the same amino acid sequence, or a sequence which retains a functional characteristic of the product encoded by the wild-type gene.

Sequences comprising changes to or differences from the sequences shown may also be employed in the present invention, as discussed herein.

According to a preferred embodiment of the present invention the nucleotide sequence originates from a species of the genus *Citrus*, such as grapefruit and pomelo.

The nucleotide sequence information provided herein or any part thereof may be used in a data-base search to find homologous sequences, expression products of which can be tested for flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase activity.

A further aspect of the present invention provides a method of identifying and cloning a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase gene homolog from a plant species other than pomelo which method employs a nucleotide sequence derived from SEQ ID NO:20. Nucleic acid libraries may be screened using techniques well known to those skilled in the art and homologous sequences thereby identified then tested. Hybridization may involve probing nucleic acid and identifying positive hybridization under suitable stringent conditions (in accordance with known techniques) and/or use of oligonucleotides as primers in a method of nucleic acid amplification, such as PCR. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridizations identified as positive which can be investigated further. It is well known in the art to increase stringency of hybridization gradually until only a few positive clones remain. As an alternative to probing, though still employing nucleic acid hybridization, oligonucleotides designed to amplify DNA sequences may be used in PCR reactions or other methods involving amplification of nucleic acid, using routine procedures. See for instance "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al. 1990, Academic Press, New York. Assessment of whether or not such a PCR product corresponds to a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase may be conducted in various ways. A PCR band from such a reaction might contain a complex mix of products. Individual products may be cloned and each one individual product may be cloned and each one individually screened. It may be analyzed by transformation to assess function on introduction into a plant of interest.

Thus, included within the scope of the present invention are polynucleotide molecules which encode amino acid sequences which are homologs of flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase of pomelo. Homology may be at the nucleotide sequence and/or amino acid sequence level. Preferably, the nucleic acid or amino acid sequence of a homolog, or a mutant, allele, derivative or variant (see above) shares homology with SEQ ID NOs:20 or 21, respectively, preferably at least about 50 %, or at least about 60 %, or at least about 70 %, or at least about 75 %, or at least about 80 % homology, most preferably at least about 90 % homology, and the encoded product shares a phenotype with the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase gene. "Homology" may be understood to refer to similarity, in functional terms, in an amino acid sequence, as is standard in the art. Thus, for example, a % similarity figure will include amino acid differences that have little or no functional significance, such as leucine to isoleucine. Otherwise, homology may be taken to refer to identity. These definitions are further elaborated hereinabove. Thus, for example, gene homologs from other members of the genus *Citrus* may be identified.

In certain embodiments, an allele, variant, derivative, mutant or homolog of the specific sequence may show little overall homology, say about 20 % or about 25 %, or about 30 %, or about 35 % or about 40 % or about 45 %, with the specific sequence. However, in functionally significant domains or regions the amino acid homology may be much higher. Deletion mutagenesis, for example, may be used to test the function of a region of the polypeptide and its role in or necessity for catalytic activity.

Also according to the invention there is provided a plant cell having incorporated into its genome a sequence of nucleotides as provided by the present invention, under operative control of a promoter for control of expression of the encoded polypeptide. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of nucleotides into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome.

The present invention further encompasses a plant comprising a plant cell comprising nucleic acid according to the present invention e.g., as a result of introduction of the nucleic acid into the cell or an ancestor thereof, and selfed or hybrid progeny and any descendant of such a plant, also any

part or propagule of such a plant, progeny or descendant, including sexually or apomictically obtained seed and vegetatively propagated plant material.

The principal characteristics which may be altered using the present invention are controlling the bitterness of fruits of plants of the genus *Citrus* as further detailed hereinunder. Over-expression of the gene product of the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase gene may lead to either bitterer fruits or less bitter fruits via a co-suppression mechanism; under-expression may lead to less bitter fruits. Down-regulation may be achieved, for example, with "gene silencing" techniques such as anti-sense or sense regulation, discussed further below. It can also be achieved with gene knockout.

This degree of control is useful to provide fruits of desired bitterness which can be used in the fresh fruit market or in the juice production industry, diminishing the need for de-bittering processes.

In preparing a vector according to the present invention various DNA fragments may be manipulated as necessary to create the desired vector or construct. This includes using linkers or adaptors as necessary to form suitable restriction sites or to eliminate unwanted restriction sites or other like manipulations which are known to those of ordinary skill in the art.

Promoters which are known or found to cause transcription of a selected gene in plant cells can be used in the present invention. Such promoters may be obtained from plants, plant pathogenic bacteria or plant viruses, and include, but are not necessarily limited to, the 35S and 19S promoters of cauliflower mosaic virus (CaMV35S and CaMV19S), the full-length transcript promoter from the figwort mosaic virus (FMV35S) and promoters isolated from plant genes such as EPSP synthase and ssRUBISCO genes, and promoters obtained from T-DNA genes of *Agrobacterium tumefaciens*, such as nopaline and mannopine synthases. The particular promoter selected should be capable of causing sufficient expression of sense or antisense RNA.

Particularly useful promoters for use in the present invention are fruit specific promoters which are expressed, for example, during ethylene production in the fruit and the full-length transcript promoter from the figwort mosaic virus (FMV35S). The FMV35S promoter is particularly useful because of its ability to cause uniform and high levels of expression.

The DNA sequence of a FMV35S promoter is presented in U.S. Pat. No. 5,512,466 and is identified as SEQ ID NO:17 therein. Examples of fruit specific promoters include the E8, E4, E17 and J49 promoters from

tomato (Lincoln, J. and Fischer, R. (1988). Diverse mechanisms for the regulation of ethylene-inducible gene expression. *Mol Gen Genet* 212, 71-75), as well as the 2A11 promoter as described in U.S. Pat. No. 4,943,674.

As used herein, the term "CaMV35S" or "FMV35S" promoter
5 include variations of these promoters, e.g., promoters derived by means of ligation with operator regions, random or controlled mutagenesis, addition or duplication of enhancer sequences, etc.

A 3' non-translated region is preferably included in a sense vector according to the present invention and includes a polyadenylation signal
10 which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of a sense RNA sequence. Examples of suitable 3' regions are the 3' transcribed, non-translated regions containing the polyadenylation signal of the tumor-inducing (Ti) plasmid genes of *Agrobacterium*, such as the nopaline synthase (NOS) gene, and plant genes
15 like the 7S soybean storage protein genes and the pea E9 small subunit of the RuBP carboxylase gene (ssRUBISCO).

A sense RNA produced by a vector of the present invention also preferably contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be
20 specifically modified so as to increase translation of the sense RNA. The 5' non-translated regions can also be obtained from viral RNA's, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to vector constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter
25 sequence. Rather, the non-translated leader sequences can be part of the 5' end of the non-translated region of the native coding sequence, or part of the promoter sequence, or can be derived from an unrelated promoter or coding sequence.

In a preferred embodiment according to the present invention, the
30 vector that is used to introduce the sense or antisense RNA into the host cells or plants will comprise an appropriate selectable marker. In a more preferred embodiment according to the present invention the vector is a plant expression vector comprising both a selectable marker and an origin of replication. In another most preferred embodiment according to the
35 present invention the vector will be a shuttle vector, which can propagate both in *E. coli* (wherein the construct comprises an appropriate selectable marker and origin of replication) and be compatible for propagation or integration in the genome of the organism of choice. In yet another

embodiment, the construct comprising the promoter of choice and the gene of interest is placed in a viral vector which is used to infect the cells. This virus may be integrated in the genome of the organism of choice or may remain non-integrated.

5 The promoter of choice that is used in conjunction with this invention will comprise any suitable promoter as further detailed herein. It will be appreciated by one skilled in the art, however, that it is necessary to make sure that the transcription start site(s) will be located upstream of the open reading frame. In a preferred embodiment of the present invention,
10 the promoter that is selected will comprise an element that is active in the particular host plant cells of interest.

These elements may be selected from transcriptional regulators that activate the transcription of genes essential for the survival of these cells in conditions of stress or starvation, including the heat shock proteins.
15 Promoters containing this type of sequence may advantageously be used according to the present invention.

As further detailed hereinabove, DNA sequences encoding the translational start site (ATG) of the gene to be expressed, will be placed downstream of the transcription start site(s). Any equivalent functional
20 element selected from similar elements in this or other organisms may be used as appropriate in the organism of choice. Equivalent functional elements will include elements with synthetic bases, or elements found in other genes of plants as well as elements found in genes of other unicellular or multicellular organisms.

25 According to one embodiment of the present invention secretion of the protein out of the cell is preferred. In this embodiment the construct will comprise a signal sequence to effect secretion as is known in the art. A signal sequence that is recognized in the active growth phase will be most preferred. As will be recognized by the skilled artisan, the appropriate
30 signal sequence should be placed immediately downstream of the translational start site (ATG), and in frame with the coding sequence of the gene to be expressed.

Introduction of the construct or vector into the cells is accomplished by any conventional method for transfection, infection or the like, as is
35 known in the art. In constructs comprising a selectable marker the cells may be selected from those bearing functional copies of the construct. If the plasmid comprising the gene of interest is episomal the appropriate selective conditions will be used during growth. Stable transfectants and stable cell

lines may be derived from the transfected cells in appropriate cases, in order to conveniently maintain the genotype of interest. Cell growth is accomplished in accordance with the cell type, using any standard growth conditions as may be suitable to support the growth of the specific cell line.

5 A DNA construct of the present invention can be inserted into the genome of a plant by any suitable method. Suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, such as those disclosed by Bevan and Chilton, Ann. Rev. Genetics 16, 357-384, 1982; U.S. Pat. No. 4,940,838 and others. In
10 addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA constructs of this invention into plant cells. Such methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, particle gun technology, and
15 transformation using viruses.

The construction of vectors capable of being inserted into a plant genome via *Agrobacterium tumefaciens* mediated delivery is known to those of ordinary skill in the art. Typical plant cloning vectors comprise selectable and scoreable marker genes, T-DNA borders, cloning sites,
20 appropriate bacterial genes to facilitate identification of transconjugates, broad host-range replication and mobilization functions and other elements as desired.

If *Agrobacterium* mediated delivery is chosen, once the vector has been introduced into the disarmed *Agrobacterium* strain, the desired plant
25 can then be transformed. Any known method of transformation that will work with the desired plant can be utilized.

Thus, further according to the present invention there are provided a plant cell and a transgenic plant of a species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, such
30 as, but not limited to, grapefruit or pomelo, wherein the plant cell or transgenic plant are genetically modified to include, in an expressible antisense orientation, a nucleotide sequence encoding an antisense RNA molecule being capable of *in vivo* base pairing with the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, to thereby render
35 the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, when expressed, amenable to degradation by nucleases present in the plant cell or transgenic plant. The plant cell or transgenic plant according to this

aspect of the present invention exhibit either very low or no flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase activity.

It will be appreciated that the nucleotide sequence encoding an antisense RNA molecule being capable of *in vivo* base pairing with the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA according to the present invention can be either extrachromosomal or intrachromosomal. In any case, as further detailed herein, the nucleotide sequence is preferably ligated to an expression vector in the antisense orientation downstream to a promoter element and optionally other gene expression control elements.

The nucleotide sequence encoding an antisense RNA molecule being capable of *in vivo* base pairing with the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA according to the present invention can be as set forth in SEQ ID NO:20 or a portion thereof longer than 10 nucleotides, preferably longer than 15 nucleotides, more preferably longer than 20 nucleotides, most preferably longer than 30 or 40 nucleotides, so as to ensure specific base pairing with the endogenous flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA produced by the plant cell under physiological conditions.

Alternatively, the nucleotide sequence encoding an antisense RNA molecule being capable of *in vivo* base pairing with the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA according to the present invention can share between 80 and 100 % identical bases with SEQ ID NO:20 or a portion thereof longer than 10 nucleotides, preferably longer than 15 nucleotides, more preferably longer than 20 nucleotides, most preferably longer than 30 or 40 nucleotides, as determined using a sequence analysis software package developed by the Genetic Computer Group (GCG) at the University of Wisconsin with gap creation penalty of 50 and gap extension penalty of 3, so as to ensure specific base pairing with the endogenous flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA produced by the plant cell.

Still alternatively, the nucleotide sequence encoding an antisense RNA molecule being capable of *in vivo* base pairing with the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA according to the present invention can be hybridizable *in vitro* with SEQ ID NO:20 or a portion thereof longer than 10 nucleotides, preferably longer than 15 nucleotides, more preferably longer than 20 nucleotides, most preferably longer than 30 or 40 nucleotides, under hybridization selected from the

group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, moderate hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C, whereas mild hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50 °C, final wash solution of 2 x SSC and final wash at 40 °C, so as to ensure specific base pairing with the endogenous flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA produced by the plant cell. These alternative hybridization conditions are useful in evaluating hybridization capabilities of sequences according to all of the aspects of the present invention. Other hybridization conditions can also be employed.

Step by step protocols for the above specified hybridizations and the mentioned other hybridizations are found in *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al. 1989, Cold Spring Harbor Laboratory Press, which is incorporated by reference as if fully set forth herein.

Further according to the present invention there is provided a cell genetically modified to include, in an expressible sense orientation, a nucleotide sequence encoding a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase. The nucleotide sequence according to this aspect of the present invention can be complementary DNA or genomic DNA, and it can be either extrachromosomal or intrachromosomal. It is preferably ligated to an expression vector in the sense orientation and has any of the features described herein above with respect to the isolated polynucleotide according to the present invention. The cell according to this aspect of the present invention can be either a microorganism (either a prokaryote or a eukaryote) or a cell of a multicellular organism, such as a cell of a multicellular plant. In any case, for reasons further detailed hereinunder, the selected cell is preferably capable of producing an activated rhamnose, either in the form of UDP-rhamnose or of dTDP-rhamnose.

Suitable microorganism which are known to produce dTDP-rhamnose include, but are not limited to, *Lactobacillus Spp.*, such as *Lactobacillus casei* and *Lactobacillus delbrueckii* and yeast such as *Saccharomyces cerevisiae*.

Cells of suitable multicellular plants include, for example, cells of plants of the genus *Citrus*, such as, but not limited to, grapefruit and pomelo, both being capable of producing UDP-rhamnose. Additional examples include cells of tobacco (*Nicotiana tabacum*), grapes (*Vitis vinifera*) and carrot (*Daucus carota*).

Further according to the present invention there is provided a plant cell or a transgenic plant of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, such as, but not limited to, grapefruit and pomelo, the plant cell or transgenic plant being genetically modified to include, in an overexpressible sense orientation, a nucleotide sequence encoding a sense RNA molecule being capable of inducing a co-suppression effect to thereby reduce production of the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the plant cell or the transgenic plant.

The transgenic plants according to the present invention, either expressing the nucleotide sequence according to the present invention as herein described in an antisense form or in an overexpressible sense form, both are characterized by reduced production of flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase. Plants can therefore be selected via taste tests to produce fruits of desired degree of bitterness, to provide, for example, less bitter fruits. Varieties with optimal perceived bitterness levels can be propagated vegetatively by land grafting or other procedures known to the ordinary artisan, and can be used to provide fruits to the fresh fruit market and to produce juices, such as grapefruit juice, that do not require debittering.

Furthermore, according to still another aspect of the present invention there is provided a transgenic plant or cell of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase. The transgenic plant or cell is genetically modified to knock-out a gene encoding the endogenous flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase thereof to thereby abolish production of flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the transgenic plant or cell.

One ordinarily skilled in the art can readily design a knock-out construct including both positive and negative selection genes for efficiently selecting transfected cells that underwent a homologous recombination event with the construct. Such cells can be cultured and propagated and can further be induced to differentiate into seedlings and grown plants, as well known in the art. Further details concerning uses and procedures of plant

gene knock-out are found in Miao et al., 1995; Strepp et al., 1998; and Cooley et al., 1998, which are incorporated by reference as if fully set forth herein.

The flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase gene
5 according to the present invention can be used in a process for enzymatically modifying a 1-6 linkage of hesperidin to a 1-2 linkage of neohesperidin by removing and re-coupling the terminal rhamnose. This is done in a two step process as shown in the left path of Figure 1. First, hesperidin from orange peels is treated with immobilized commercial
10 hesperidinase to remove the 1-6 linkage. This process is already used commercially as a de-bittering step for citrus juice. The second step involves using a transgenic organism heavily expressing the gene to re-rhamnosylate H7G in the correct 1-2 position. The organism must be capable of naturally producing copious quantities of UDP- or dTDP-
15 rhamnose.

Thus, further according to the present invention, there is provided a commercial process of producing neohesperidin. The process is effected by implementing the following steps, in which, in a first step, hesperidin is obtained, for example, by extraction from a hesperidin producing species of
20 the genus *Citrus* such as orange. Then, the hesperidin is treated with a hesperidinase, thereby hesperetin-7-glucoside is obtained. Thereafter, the hesperetin-7-glucoside is treated with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose, such as UDP-rhamnose or dTDP-rhamnose, and thereby neohesperidin is obtained.

25 Further according to the present invention there is provided a commercial process of producing neohesperidin dihydrochalcone. The process is effected by implementing the following steps, in which, in a first step, hesperidin is obtained, for example, by extraction from a hesperidin producing species of the genus *Citrus* such as orange. Then, the hesperidin
30 is treated with a hesperidinase, thereby hesperetin-7-glucoside is obtained. Thereafter, the hesperetin-7-glucoside is treated with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose, thereby neohesperidin is obtained. Subsequently, the neohesperidin is treated with an alkali, thereby neohesperidin chalcone is
35 obtained. Finally, the neohesperidin chalcone is reduced, e.g., by a Pd catalyzed H₂ surface reduction process, thereby neohesperidin dihydrochalcone is obtained.

Preferably, the hesperidinase is immobilized on a solid support, whereas the hesperidin is treated with the hesperidinase while passing over the solid support.

Treating the hesperetin-7-glucoside with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose is preferably effected *in vivo* by a cell genetically modified to overexpress the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, wherein the cell selected produces activated rhamnose and is capable of intake of the hesperetin-7-glucoside. The neohesperidin is then extracted from the cell. Preferably, the neohesperidin is extracted from the cell prior to the treatment thereof with the alkali. The cell can be of a bacterium, a fungus, a yeast or of a higher plant, as long as it is capable of generating activated rhamnose and is capable of intake of the hesperetin-7-glucoside. The cell can be of a microorganism or of a higher plant. Suitable microorganisms include bacteria and yeast cells. *Lactobacillus casei*, *Lactobacillus delbrueckii* and *Saccharomyces cerevisiae* provide good examples for microorganisms. Tobacco (*Nicotiana tabacum*), grapes (*Vitis vinifera*) and carrot (*Daucus carota*) provide good examples for higher plant cells which are readily propagated in culture and qualify with the required criteria.

This aspect of the present invention is of high significance. It is well known that *in vitro* formation of activated sugar moieties is highly inefficient. Therefore, only when the supply of activated rhamnose is effected *in vivo* by an organism capable also of intake of hesperetin-7-glucoside, either naturally or rendered capable thereof by physical, chemical, biological or genetic means, and of overexpressing flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in stoichiometric amounts, would the process described herein be amenable to industrialization.

Further according to the present invention there is provided a method of modifying a rhamnose-1-6-glucose linkage of a chemical compound to a rhamnose-1-2-glucose linkage. The method is effected by implementing the following method steps, in which, in a first step, for removal of the rhamnose group, the chemical compound is enzymatically treated, thereby a first derivative of the chemical compound having a terminal glucose group is obtained. Then, the first derivative is enzymatically treated with recombinant flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose, thereby a second derivative chemical compound having the rhamnose-1-2-glucose linkage is obtained. Enzymatically treating the first derivative with a flavanone-7-*O*-glucoside-

2"-O-rhamnosyl-transferase in the presence of activated rhamnose is preferably effected *in vivo* by a cell genetically modified to overexpress the flavanone-7-O-glucoside-2"-O-rhamnosyl-transferase, wherein the cell produces activated rhamnose and is capable of intake of the first derivative.

5 The second derivative can be extracted from the cell.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

20 *Isolation of a gene encoding a flavanone-7-O-glucoside-2"-O-rhamnosyl-transferase.*

Previous attempts to isolate the gene based on purified protein were unsuccessful. These included cDNA library screens using oligonucleotides as well as antibodies. The successful approach, RT-PCR using degenerate primers followed by RACE is described herein.

A cDNA expression library of pomelo young leaves was constructed in Lambda-gt11. Antibodies prepared against pomelo 1-2 rhamnosyl-transferase were used to screen this library. Positive-looking clones were later found to be artifacts in that they did not contain a relevant sequence.

Several oligonucleotides were designed according to the partial peptide sequence and were used to screen the above library by standard techniques. Any resulting clones were irrelevant.

Thus, isolation of the gene encoding flavanone-7-O-glucoside-2"-O-rhamnosyl-transferase (1-2 rhamnosyl-transferase) involved a lengthy process initiated in the purification to homogeneity of the protein, followed by partial peptide sequencing and finalized by cDNA isolation based on RT-PCR and RACE. Details of the process are as follows:

Purification of the enzyme and activity assay: Purification and functional analysis of the flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase protein from young pomelo leaves was as described (Bar-Peled et al. 1991).

5 **Partial peptide sequence of the protein:** The purified protein was subject to partial proteolysis, using several different proteases (Lys-C; Asp-N; Trypsin), followed by separation of peptides by SDS-PAGE and blotting on PVDF membranes. Partial peptide sequences generated by Edman degradation were as follows (only conclusive sequence data is given; amino
10 acids in question are not shown): Lys-C cleavage yielded two partial sequences: I N Y F L H L T A (SEQ ID NO:1) and Y P F (SEQ ID NO:2); Asp-N cleavage yielded four partial sequences: I A A I L F L (SEQ ID NO:3), Y F P S L M G N (SEQ ID NO:4), E K M T I E E A (SEQ ID NO:5) and L F Q P (SEQ ID NO:6); whereas, trypsin cleavage yielded a
15 single partial sequence: V V D N G M G M V V P R D K (SEQ ID NO:7)

Isolation of partial cDNA fragments: Partial cDNA fragments were obtained by an RT-PCR approach on target tissue mRNA (young pomelo leaves) using degenerate primers based on the partial peptide sequences. Pomelo young leaf single strand cDNA was prepared by reverse
20 transcription of poly-A RNA using a PCR-adapted poly-T primer (primer contains sequences identical to the -40M13 forward primer followed by 15 T's; see below). PCR was used to amplify gene specific fragments using degenerate primers (designed according to partial peptide sequences; see below) in conjunction with the -40M13 forward primer. Primer sequences
25 were as follows:

 Gene specific degenerate primers were: 5'-GAT(C)AAT(C)GGIAT GGGIATGGT-3' (SEQ ID NO:8) for D N G M G M V (SEQ ID NO:9); 5'-GAA(G)AAG(A)ATGACIATT(CA)GAA(G)GAA(G)GC-3' (SEQ ID NO:10) for E K M T I E E A (SEQ ID NO:5); and 5'-
30 AAC(T)TAC(T)TTC(T) CTICAC(T)CTIACIGC-3' (SEQ ID NO:11) for N Y F L H L T A (SEQ ID NO:12).

 General primers were: PCR-adapted poly-T primer 5'-GTTTTCCCA GTCACGACGTTTTTTTTTTTTTTTTT (SEQ ID NO:13); and -40M13 forward primer 5'-GTTTTCCCAGTCACGACG-3' (SEQ ID NO:14).

35 Amplified DNA fragments were separated and isolated from agarose gels and then cloned into pGEM-T vector (Promega, Madison WI). Sequence was obtained by automated sequencing using universal primers (T7 and SP6).

Completion of cDNA sequence: The full length cDNA sequence was obtained after isolating the missing 5' segment by 5'-RACE (Schaefer B.C., 1995) using the following gene specific primers which were derived from the partial cDNA sequence data: RACE1: 5'-
5 CATGCCCATACCATTGTC-3' (SEQ ID NO:15); RACE2: 5'-
GACAATGGTATGGGCATG-3' (SEQ ID NO:16); and RACE3: 5'-
CCTCAACCACCGAGCCCCAACCAC-3' (SEQ ID NO:17).

Based on the total assembled sequence data, the complete coding region was isolated by RT-PCR using a proof-reading thermostable
10 polymerase (Pfu; Stratagene, La-Jolla, CA) and the following primers (each primer contains a cloning adapter sequence; gene specific sequences are underlined): 5'-CATCTAGAATGGATACCAAGCATCAAG-3' (SEQ ID NO:18); and 5'-CAGGATCCTTATTCAGATTCTTGACAAG-3' (SEQ ID NO:19).

15 The resulting translated coding region (1359 base-pairs; Figure 2, SEQ ID NO: 20) contains all partial peptide sequences previously determined by protein sequencing. The sequence of the protein encoded by SEQ ID NO: 20 is given in SEQ ID NO:21.

20 Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended
25 claims.

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WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide having a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase catalytic activity.
2. The isolated polynucleotide of claim 1, wherein said nucleotide sequence is selected from the group consisting of DNA and RNA.
3. The isolated polynucleotide of claim 1, wherein said nucleotide sequence is selected from the group consisting of complementary DNA, genomic DNA and messenger RNA.
4. The isolated polynucleotide of claim 1, further comprising a vector, wherein said nucleotide sequence is ligated to said vector.
5. The isolated polynucleotide of claim 4, wherein said vector is an expression vector.
6. The isolated polynucleotide of claim 5, wherein said nucleotide sequence is ligated to said vector in an orientation selected from the group consisting of a sense orientation and an antisense orientation.
7. The isolated polynucleotide of claim 5, wherein said vector is selected from the group consisting of a vector propagatable in plant cells and a vector propagatable in a microorganism cell.
8. The isolated polynucleotide of claim 5, wherein said nucleotide sequence is as set forth in SEQ ID NO:20 or a functional part thereof.
9. The isolated polynucleotide of claim 1, wherein said nucleotide sequence shares between 50 and 100 % identical bases with SEQ ID NO:20 or a functional part thereof, as determined using a sequence analysis software package developed by the Genetic Computer Group

(GCG) at the University of Wisconsin with gap creation penalty of 50 and gap extension penalty of 3.

10. The isolated polynucleotide of claim 1, wherein said nucleotide sequence is hybridizable with SEQ ID NO:20 or a functional part thereof under hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, moderate hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C, whereas mild hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50 °C, final wash solution of 2 x SSC and final wash at 40 °C.

11. The isolated polynucleotide of claim 1, wherein said polypeptide is as set forth in SEQ ID NO:21 or a functional part thereof.

12. The isolated polynucleotide of claim 1, wherein said polypeptide shares between 50 and 100 % identical or conserved amino acids with SEQ ID NO:21 or a functional part thereof, as determined using a sequence analysis software package developed by the Genetic Computer Group (GCG) at the University of Wisconsin with gap creation penalty of 12 and gap extension penalty of 4.

13. The isolated polynucleotide of claim 1, wherein said nucleotide sequence originates from a species of the genus *Citrus*.

14. A plant cell of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, wherein the plant cell is genetically modified to include, in an expressible antisense orientation, a nucleotide sequence encoding an antisense RNA molecule being capable of *in vivo* base pairing with said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, to thereby render said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, when expressed, amenable to degradation by nucleases present in the plant cell.

15. The plant cell of claim 14, wherein said nucleotide sequence is selected from the group consisting of a complementary DNA and a genomic DNA.

16. The plant cell of claim 14, wherein said nucleotide sequence is extrachromosomal.

17. The plant cell of claim 14, wherein said nucleotide sequence is intrachromosomal.

18. The plant cell of claim 14, wherein said nucleotide sequence is ligated to an expression vector in antisense orientation.

19. The plant cell of claim 18, wherein said nucleotide sequence is as set forth in SEQ ID NO:20 or a portion thereof longer than 15 nucleotides.

20. The plant cell of claim 14, wherein said nucleotide sequence shares between 80 and 100 % identical bases with SEQ ID NO:20 or a portion thereof longer than 15 nucleotides, as determined using a sequence analysis software package developed by the Genetic Computer Group (GCG) at the University of Wisconsin with gap creation penalty of 50 and gap extension penalty of 3.

21. The plant cell of claim 14, wherein said nucleotide sequence is hybridizable *in vitro* with SEQ ID NO:20 or a portion thereof longer than 15 nucleotides under hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, moderate hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C, whereas mild hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50 °C, final wash solution of 2 x SSC and final wash at 40 °C.

22. The plant cell of claim 14, wherein said nucleotide sequence originates from a species of the genus *Citrus*.

23. The plant cell of claim 14, wherein said species is selected from the group consisting of grapefruit and pomelo.

24. A transgenic plant of a species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, wherein the transgenic plant is genetically modified to include, in an expressible antisense orientation, a nucleotide sequence encoding an antisense RNA molecule being capable of *in vivo* base pairing with said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, to thereby render said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase messenger RNA, when expressed, amenable to degradation by nucleases present in the transgenic plant.

25. The transgenic plant of claim 24, wherein said nucleotide sequence is selected from the group consisting of a complementary DNA and a genomic DNA.

26. The transgenic plant of claim 24, wherein said nucleotide sequence is extrachromosomal.

27. The transgenic plant of claim 24, wherein said nucleotide sequence is intrachromosomal.

28. The transgenic plant of claim 24, wherein said nucleotide sequence is ligated to an expression vector in antisense orientation.

29. The transgenic plant of claim 28, wherein said nucleotide sequence is as set forth in SEQ ID NO:20 or a portion thereof longer than 15 nucleotides.

30. The transgenic plant of claim 24, wherein said nucleotide sequence shares between 80 and 100 % identical bases with SEQ ID NO:20 or a portion thereof longer than 15 nucleotides, as determined using the sequence analysis software package developed by the Genetic Computer

Group (GCG) at the University of Wisconsin with gap creation penalty of 50 and gap extension penalty of 3.

31. The transgenic plant of claim 24, wherein said nucleotide sequence is hybridizable *in vitro* with SEQ ID NO:20 or a portion thereof longer than 15 nucleotides under hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, moderate hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C, whereas mild hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50 °C, final wash solution of 2 x SSC and final wash at 40 °C.

32. The transgenic plant of claim 24, wherein said nucleotide sequence originates from a species of the genus *Citrus*.

33. The transgenic plant of claim 24, wherein said species is selected from the group consisting of grapefruit and pomelo.

34. A cell genetically modified to include, in an expressible sense orientation, a nucleotide sequence encoding a flavanone-7-O-glucoside-2"-O-rhamnosyl-transferase.

35. The cell of claim 34, wherein said nucleotide sequence is selected from the group consisting of complementary DNA and genomic DNA.

36. The cell of claim 34, wherein said nucleotide sequence is extrachromosomal.

37. The cell of claim 34, wherein said nucleotide sequence is intrachromosomal.

38. The cell of claim 34, wherein said nucleotide sequence is ligated to an expression vector in sense orientation.

39. The cell of claim 38, wherein said nucleotide sequence is as set forth in SEQ ID NO:20 or an active part thereof.

40. The cell of claim 34, wherein said nucleotide sequence shares between 50 and 100 % identical bases with SEQ ID NO:20 or an active part thereof, as determined using the sequence analysis software package developed by the Genetic Computer Group (GCG) at the University of Wisconsin with gap creation penalty of 50 and gap extension penalty of 3.

41. The cell of claim 34, wherein said nucleotide sequence is hybridizable *in vitro* with SEQ ID NO:20 or an active part thereof under hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, moderate hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C, whereas mild hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50 °C, final wash solution of 2 x SSC and final wash at 40 °C.

42. The cell of claim 34, wherein said nucleotide sequence originates from a species of the genus *Citrus*.

43. The cell of claim 34, wherein the cell is of a microorganism.

44. The cell of claim 43, wherein said microorganism is producing activated rhamnose.

45. The cell of claim 43, wherein said microorganism is selected from the group consisting of *Lactobacillus* and *Saccharomyces*.

46. The cell of claim 34, wherein the cell is of a plant species.

47. The cell of claim 46, wherein said plant species is of a genus selected from the group consisting of *Citrus*, *Nicotiana*, *Vitis* and *Daucus*.

48. A commercial process of producing neohesperidin comprising the steps of:

- (a) obtaining hesperidin;
- (b) treating said hesperidin with a hesperidinase, thereby obtaining hesperetin-7-glucoside; and
- (c) treating said hesperetin-7-glucoside with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose, thereby obtaining neohesperidin.

49. The commercial process of claim 48, wherein said activated rhamnose is selected from the group consisting of UDP-rhamnose or dTDP-rhamnose.

50. The commercial process of claim 48, wherein said hesperidinase is immobilized on a solid support, whereas said hesperidin is treated with said hesperidinase while passing over said solid support.

51. The commercial process of claim 48, wherein said treatment of hesperetin-7-glucoside with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in presence of activated rhamnose of step (c) is effected *in vivo* by a cell genetically modified to overexpress said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, which said cell is producing activated rhamnose and is capable of intake of said hesperetin-7-glucoside.

52. The commercial process of claim 51, further comprising the step of extracting said neohesperidin from said cell.

53. A commercial process of producing neohesperidin dihydrochalcone comprising the steps of:

- (a) obtaining hesperidin;
- (b) treating said hesperidin with a hesperidinase, thereby obtaining hesperetin-7-glucoside;
- (c) treating said hesperetin-7-glucoside with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the presence of activated rhamnose, thereby obtaining neohesperidin;
- (d) treating said neohesperidin with an alkali, thereby obtaining neohesperidin chalcone; and

- (e) reducing said neohesperidin chalcone, thereby obtaining neohesperidin dihydrochalcone

54. The commercial process of claim 53, wherein said activated rhamnose is selected from the group consisting of UDP-rhamnose or dTDP-rhamnose.

55. The commercial process of claim 53, wherein said hesperidinase is immobilized on a solid support, whereas said hesperidin is treated with said hesperidinase while passing over said solid support.

56. The commercial process of claim 53, wherein said treatment of hesperetin-7-glucoside with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in presence of activated rhamnose of step (c) is effected *in vivo* by a cell genetically modified to overexpress said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, which said cell is producing activated rhamnose and is capable of intake of said hesperetin-7-glucoside.

57. The commercial process of claim 56, further comprising the step of extracting said neohesperidin from said cell prior to treating said neohesperidin with said alkali.

58. A method of modifying a rhamnose-1-6-glucose linkage of a chemical compound to a rhamnose-1-2-glucose linkage comprising the steps of:

- (a) enzymatically treating said chemical compound to remove a rhamnose group thereof, thereby obtaining a first derivative chemical compound having a terminal glucose group; and
- (b) enzymatically treating said first derivative with recombinant flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in a presence of activated rhamnose, thereby obtaining a second derivative chemical compound having said rhamnose-1-2-glucose linkage.

59. The method of claim 58, wherein said activated rhamnose is selected from the group consisting of UDP-rhamnose or dTDP-rhamnose.

60. The method of claim 58, wherein enzymatically treating said first derivative with a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in said presence of activated rhamnose is effected *in vivo* by a cell genetically modified to overexpress said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, which said cell is producing activated rhamnose and is capable of intake of said first derivative.

61. The method of claim 60, further comprising the step of extracting said second derivative from said cell.

62. A plant cell of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, wherein the plant cell is genetically modified to include, in an overexpressible sense orientation, a nucleotide sequence encoding a sense RNA molecule being capable of inducing a co-suppression effect to thereby reduce production of said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the plant cell.

63. The plant cell of claim 62, wherein said nucleotide sequence is selected from the group consisting of a complementary DNA and a genomic DNA.

64. The plant cell of claim 62, wherein said nucleotide sequence is extrachromosomal.

65. The plant cell of claim 62, wherein said nucleotide sequence is intrachromosomal.

66. The plant cell of claim 62, wherein said nucleotide sequence is ligated to an expression vector in sense orientation.

67. The plant cell of claim 66, wherein said nucleotide sequence is as set forth in SEQ ID NO:20 or an active part thereof.

68. The plant cell of claim 62, wherein said nucleotide sequence shares between 50 and 100 % identical bases with SEQ ID NO:20 or an active part thereof, as determined using a sequence analysis software package developed by the Genetic Computer Group (GCG) at the

University of Wisconsin with gap creation penalty of 50 and gap extension penalty of 3.

69. The plant cell of claim 62, wherein said nucleotide sequence is hybridizable *in vitro* with SEQ ID NO:20 or an active part thereof under hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, moderate hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C, whereas mild hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50 °C, final wash solution of 2 x SSC and final wash at 40 °C.

70. The plant cell of claim 62, wherein said nucleotide sequence originates from a species of the genus *Citrus*.

71. The plant cell of claim 62, wherein said species is selected from the group consisting of grapefruit and pomelo.

72. A transgenic plant of a species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, wherein the plant is genetically modified to include, in an overexpressible sense orientation, a nucleotide sequence encoding a sense RNA molecule being capable of inducing a co-suppression effect to thereby reduce production of said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the plant cell.

73. The transgenic plant of claim 72, wherein said nucleotide sequence is selected from the group consisting of a complementary DNA and a genomic DNA.

74. The transgenic plant of claim 72, wherein said nucleotide sequence is extrachromosomal.

75. The transgenic plant of claim 72, wherein said nucleotide sequence is intrachromosomal.

76. The transgenic plant of claim 72, wherein said nucleotide sequence is ligated to an expression vector in sense orientation.

77. The transgenic plant of claim 76, wherein said nucleotide sequence is as set forth in SEQ ID NO:20 or an active part thereof.

78. The transgenic plant of claim 72, wherein said nucleotide sequence shares between 50 and 100 % identical bases with SEQ ID NO:20 or an active part thereof, as determined using a sequence analysis software package developed by the Genetic Computer Group (GCG) at the University of Wisconsin with gap creation penalty of 50 and gap extension penalty of 3.

79. The transgenic plant of claim 72, wherein said nucleotide sequence is hybridizable *in vitro* with SEQ ID NO:20 or an active part thereof under hybridization selected from the group consisting of stringent hybridization, moderate hybridization and mild hybridization, wherein stringent hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 65 °C, final wash solution of 0.1 x SSC and final wash at 60 °C, moderate hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 58 °C, final wash solution of 0.5 x SSC and final wash at 50 °C, whereas mild hybridization is effected by a hybridization solution of 6 x SSC and 1 % SDS, hybridization temperature of 50 °C, final wash solution of 2 x SSC and final wash at 40 °C.

80. The transgenic plant of claim 72, wherein said nucleotide sequence originates from a species of the genus *Citrus*.

81. The transgenic plant of claim 72, wherein said species is selected from the group consisting of grapefruit and pomelo.

82. A plant cell of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, the plant cell being genetically modified to knock-out a gene encoding said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase to thereby abolish production of said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the plant cell.

83. A transgenic plant of a plant species naturally expressing a flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase, the transgenic plant being genetically modified to knock-out a gene encoding said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase to thereby abolish production of said flavanone-7-*O*-glucoside-2"-*O*-rhamnosyl-transferase in the transgenic plant.

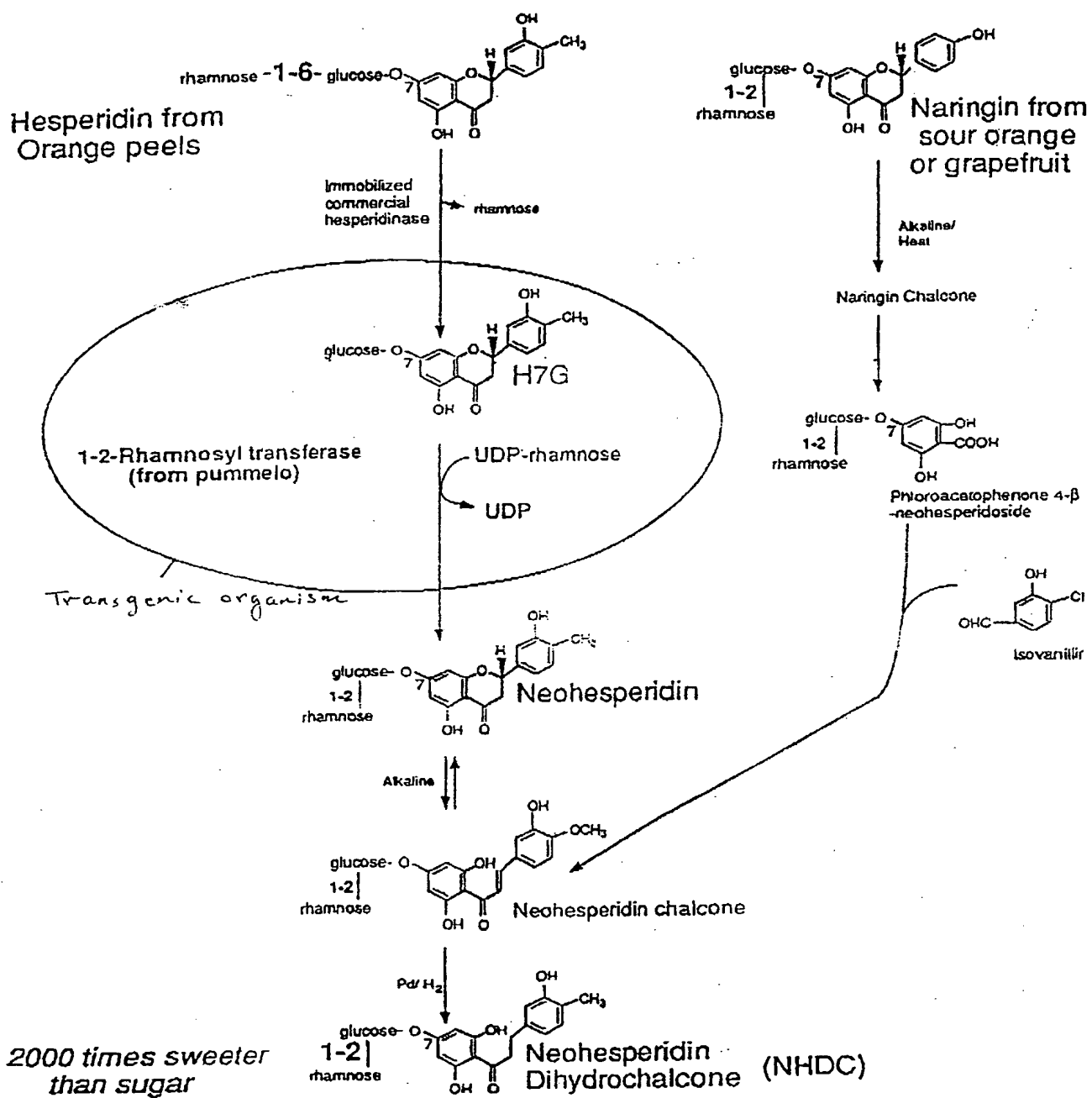


Fig. 1

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ATGGATACCAAGCATCAAGATAAGCCAAGCATTCTCATGTTACCATGGCTAGCTCATGGG 60
M D T K H Q D K P S I L M L P W L A H G
CACATAGCTCCACACCTTGAACCTTGCCAAGAAGCTTTCACAGAAAACTTCCACATATAT 120
H I A P H L E L A K K L J Q K N F H I Y
TTCTGCTCTACTCCCAACAATCTACAATCCTTCGGCAGAAATGTTGAAAAAACTTCTCA 180
F C S T P N N L Q S F G R N V E K N F S
TCITCAATACAACCTATAGAACTGCAACTTCCCAATACATTCCCTGAACCTTCCCTTCACAA 240
S S I Q L I E L Q L P N T F P E L P S Q
AATCAGACCACAAAAACCTTCTCTCCCATCTTATTATATACTCTCGTGGGAGCATTTGAA 300
N Q T T K N L P P H L I Y T L V G A F E
GACGCAAAACCTGCTTTTTTGCAACATCTTGGAGACGCTTAAACCAACCTTGTATGTAT 360
D A K P A F C N I L E T L K P T L V M Y
GATTGTGTTCCAACCGATGGCGGCGGAGGCAGCTTACCAGTATGACATAGCTGCTATTTTG 420
D L F Q P M A A E A A Y Q Y D I A A I L
TTCTTACCCTTATCTGCAGTAGCCTGCTCTTTCTTGCTGCACAATATCGTAAATCCCAGC 480
F L P L S A V A C S F L L H N I V N P S
CTGAAATACCCTTTCTTTGAATCTGATTACCAAGATAGAGAAAGCAAGAACATCAATTAC 540
L K Y P F F E S D Y Q D R E S K N I N Y
TTCTGTCATCTTACTGCCAATGGCACCTTAAACAAGACAGGTTCTTAAAGCTTTTCGAA 600
F L H L T A N G T L N K D R F L K A F E
CTATCTTGCAAATTTGTGTTTCATCAAAACATCAAGAGAGATTGAATCCAAGTACTTGGAT 660
L S C K F V F I K T S R E I E S K Y L D
TATTTTCTTCTTTAATGGGAAATGAAATAATCCAGTAGGGCCTCTAATCCAAGAACCT 720
Y F P S L M G N E I I P V G P L I Q E P
ACCTTCAAGGTAGATGATACAAAGATCATGGACTGGCTGAGCCAAAAGGAGCCTCGTTCA 780
T F K V D D T K I M D W L S Q K E P R S
GTCGTGTATGCATCCTTTGGCAGTGAGTACTTTCTTCCACGGATGAAATACATGACATA 840
V V Y A S F G S E Y F P S T D E I H D I
GCTATTGGGTTATTGCTCACCGAGGTTAATTTTATATGGGCTTTTCAGATTACATCCTGAT 900
A I G L L L T E V N F I W A F R L H P D
GAGAAAATGACGATAGAGGAAGCACTGCCTCAGGGCTTTGCTGAGGAGATTGAAAGGAAT 960
E K M T I E E A L P Q G F A E E I E R N
AATAAGGGAATGATAGTACAAGGTTGGGTTCCGCAGGCTAAAATTTTAAGGCATGGAAGC 1020
N K G M I V Q G W V P Q A K I L R H G S
ATCGGCGGATTTTTGAGTCATTGTGTTGGGGCTCGGTGGTTGAGGGGATGTTTTCGGG 1080
I G G F L S H C G W G S V V E G M V F G
GTACCAATCATAGGTGTGCCAATGGCATATGAGCAGCCAAGCAATGCCAAGGTGGTGGTT 1140
V P I I G V P M A Y E Q P S N A K V V V
GACAATGGTATGGGCATGGTCGTTCCAAGAGATAAGATCAATCAAAGACTTGGAGGAGAG 1200
D N G M G M V V P R D K I N Q R L G G E
GAGGTGGCGAGGGTCATTAAACATGTTGTGCTGCAAGAAGAAGCGAAGCAAATAAGAAGA 1260
E V A R V I K H V V L Q E E A K Q I R R
AAAGCTAATGAAATTAGTGAGAGTATGAAGAAGATAGGGGACGCACAGATGAGTGTGGTG 1320
K A N E I S E S M K K I G D A Q M S V V
GTGGAGAACTGCTGCAGCTTGTCAAGAAATCTGAATAA 1359
V E K L L Q L V K K S E *

Fig. 2

JC18 Rec'd PCT/P 2 0 JUL 2001

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: YEDA RESEARCH AND DEVELOPMENT CO. LTD
- (ii) TITLE OF INVENTION: RHAMNOSYL-TRANSFERASE GENE AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 21
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Mark M. Friedman c/o Anthony Castorina
- (B) STREET: 2001 Jefferson Davis Highway, Suite 207
- (C) CITY: Arlington
- (D) STATE: Virginia
- (E) COUNTRY: United States of America
- (F) ZIP: 22202
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 1.44 megabyte, 3.5" microdisk
- (B) COMPUTER: Twinhead® Slimnote-890TX
- (C) OPERATING SYSTEM: MS DOS version 6.2,
Windows version 3.11
- (D) SOFTWARE: Word for Windows version 2.0 converted to
an ASCII file
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Friedman, Mark M.
- (B) REGISTRATION NUMBER: 33,883
- (C) REFERENCE/DOCKET NUMBER: 910/8
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 972-3-5625553
- (B) TELEFAX: 972-3-5625554
- (C) TELEX:
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 8
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- Asn Tyr Phe Leu His Leu Thr Ala
5 8
- (2) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 3
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- Tyr Pro Phe
3
- (2) INFORMATION FOR SEQ ID NO:3:

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2

- (i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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5 7
- (2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
Tyr Phe Pro Ser Leu Met Gly Asn
5 8
- (2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
Glu Lys Met Thr Ile Glu Glu Ala
5 8
- (2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
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4
- (2) INFORMATION FOR SEQ ID NO:7:
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(A) LENGTH: 14
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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- (2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(A) LENGTH: 7
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
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5 7

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
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(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
AAXTAXTTXC TICAXCTIAC XGC 23

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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
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5 8

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
CATGCCCATTA CCATTGTC 18

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
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(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(A) LENGTH: 29
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
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(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1359
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
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CACATAGCTC CACACCTTGA ACTTGCCAAG AAGCTTTTAC AGAAAACTT CCACATATAT 120
TTCTGCTCTA CTCCAACAA TCTACAATCC TTCGGCAGAA ATGTTGAAAA AAACCTTCTCA 180
TCTTCAATAC AACTCATAGA ACTGCAACTT CCCAATACAT TCCCTGAACT TCCTTCACAA 240
AATCAGACCA CAAAAAACCT TCCTCCCCAT CTTATTTATA CTCTCGTGGG AGCATTTGAA 300

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GACGCAAAAC CTGCTTTTTG CAACATCTTG GAGACGCTTA AACCAACCCT TGTATGTAT 360
 GATTTGTTCC AACCGATGGC GCGGGAGGCA GCTTACCACT ATGACATAGC TGCTATTTTG 420
 TTCTTACCCT TATCTGCAGT AGCCTGCTCT TTCTTGCTGC ACAATATCGT AAATCCCAGC 480
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 TTCCTGCATC TTAGTGCCAA TGGCACCTTA AACAAAGACA GGTCTTAAA AGCTTTCGAA 600
 CTATCTTGCA AATTTGTGTT CATCAAAACA TCAAGAGAGA TTGAATCCAA GTACTTGGAT 660
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 GAGGTGGCGA GGGTCATTAA ACATGTTGTG CTGCAAGAAG AAGCGAAGCA AATAAGAAGA 1260
 AAAGCTAATG AAATTAGTGA GAGTATGAAG AAGATAGGGG ACGCACAGAT GAGTGTGGTG 1320
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(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 452
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

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 Trp Leu Ala His Gly His Ile Ala Pro His Leu Glu Leu Ala Lys
 20 25 30
 Lys Leu Ser Gln Lys Asn Phe His Ile Tyr Phe Cys Ser Thr Pro
 35 40 45
 Asn Asn Leu Gln Ser Phe Gly Arg Asn Val Glu Lys Asn Phe Ser
 50 55 60
 Ser Ser Ile Gln Leu Ile Glu Leu Gln Leu Pro Asn Thr Phe Pro
 65 70 75
 Glu Leu Pro Ser Gln Asn Gln Thr Thr Lys Asn Leu Pro Pro His
 80 85 90
 Leu Ile Tyr Thr Leu Val Gly Ala Phe Glu Asp Ala Lys Pro Ala
 95 100 105
 Phe Cys Asn Ile Leu Glu Thr Leu Lys Pro Thr Leu Val Met Tyr
 110 115 120
 Asp Leu Phe Gln Pro Met Ala Ala Glu Ala Ala Tyr Gln Tyr Asp
 125 130 135
 Ile Ala Ala Ile Leu Phe Leu Pro Leu Ser Ala Val Ala Cys Ser
 140 145 150
 Phe Leu Leu His Asn Ile Val Asn Pro Ser Leu Lys Tyr Pro Phe
 155 160 165
 Phe Glu Ser Asp Tyr Gln Asp Arg Glu Ser Lys Asn Ile Asn Tyr
 170 175 180
 Phe Leu His Leu Thr Ala Asn Gly Thr Leu Asn Lys Asp Arg Phe
 185 190 195
 Leu Lys Ala Phe Glu Leu Ser Cys Lys Phe Val Phe Ile Lys Thr
 200 205 210
 Ser Arg Glu Ile Glu Ser Lys Tyr Leu Asp Tyr Phe Pro Ser Leu
 215 220 225
 Met Gly Asn Glu Ile Ile Pro Val Gly Pro Leu Ile Gln Glu Pro

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| | | | |
|-------------------------------------|-----|-----|-----|
| Thr Phe Lys Val | 230 | 235 | 240 |
| Asp Asp Thr Lys Ile | 245 | 250 | 255 |
| Lys Glu Pro Arg Ser Val Val Tyr Ala | 260 | 265 | 270 |
| Phe Pro Ser Thr Asp Glu Ile His Asp | 275 | 280 | 285 |
| Leu Thr Glu Val Asn Phe Ile Trp Ala | 290 | 295 | 300 |
| Glu Lys Met Thr Ile Glu Glu Ala Leu | 305 | 310 | 315 |
| Glu Ile Glu Arg Asn Asn Lys Gly Met | 320 | 325 | 330 |
| Pro Gln Ala Lys Ile Leu Arg His Gly | 335 | 340 | 345 |
| Ser His Cys Gly Trp Gly Ser Val Val | 350 | 355 | 360 |
| Val Pro Ile Ile Gly Val Pro Met Ala | 365 | 370 | 375 |
| Ala Lys Val Val Val Asp Asn Gly Met | 380 | 385 | 390 |
| Asp Lys Ile Asn Gln Arg Leu Gly Gly | 395 | 400 | 405 |
| Ile Lys His Val Val Leu Gln Glu Glu | 410 | 415 | 420 |
| Lys Ala Asn Glu Ile Ser Glu Ser Met | 425 | 430 | 435 |
| Gln Met Ser Val Val Val Glu Lys Leu | 440 | 445 | 450 |
| Ser Glu | | | |
| 452 | | | |

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
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| | | | (43) International Publication Date: 27 July 2000 (27.07.00) |
| (21) International Application Number: PCT/IL00/00038 | | | (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). |
| (22) International Filing Date: 20 January 2000 (20.01.00) | | | |
| (30) Priority Data: 128193 22 January 1999 (22.01.99) IL | | | |
| (71) Applicants (for all designated States except US): YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL/IL]; Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL). STATE OF ISRAEL - MINISTRY OF AGRICULTURE [IL/IL]; The Volcani Center, P.O. Box 6, 50200 Bet Dagan (IL). | | | |
| (72) Inventors; and (75) Inventors/Applicants (for US only): GRESSEL, Jonathan [IL/IL]; Hayarden Street 15, 76604 Rehovot (IL). EYAL, Yoram [IL/IL]; Habrigada Street 21, 76228 Rehovot (IL). FLUHR, Robert [US/IL]; Ben-Yehuda Street 19/3, 76301 Rehovot (IL). | | | |
| (74) Agent: FRIEDMAN, Mark, M.; Beit Samueloff, Haomanim Street 7, 67897 Tel Aviv (IL). | | | Published <i>With international search report.</i> |
| | | | (88) Date of publication of the international search report: 28 September 2000 (28.09.00) |

(54) Title: RHAMNOSYL-TRANSFERASE GENE AND USES THEREOF**(57) Abstract**

An isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide having a flavanone- 7-O-glucoside-2"-O-rhamnosyl- transferase catalytic activity and its uses.

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| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | | | TR | Turkey |
| BG | Bulgaria | HU | Hungary | ML | Mali | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MN | Mongolia | UA | Ukraine |
| BR | Brazil | IL | Israel | MR | Mauritania | UG | Uganda |
| BY | Belarus | IS | Iceland | MW | Malawi | US | United States of America |
| CA | Canada | IT | Italy | MX | Mexico | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NE | Niger | VN | Viet Nam |
| CG | Congo | KE | Kenya | NL | Netherlands | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NO | Norway | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | NZ | New Zealand | | |
| CM | Cameroon | | | PL | Poland | | |
| CN | China | KR | Republic of Korea | PT | Portugal | | |
| CU | Cuba | KZ | Kazakhstan | RO | Romania | | |
| CZ | Czech Republic | LC | Saint Lucia | RU | Russian Federation | | |
| DE | Germany | LI | Liechtenstein | SD | Sudan | | |
| DK | Denmark | LK | Sri Lanka | SE | Sweden | | |
| EE | Estonia | LR | Liberia | SG | Singapore | | |

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL00/00038

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 5/14, 15/09, 15/11, 15/29, 15/54, 15/63, 15/82; A01H 5/00

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/278, 286, 295, 316; 536/23.1, 23.2, 23.6, 24.5; 435/69.1, 419, 468

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, DIALOG, WEST2.0

search terms: UDP-rhamnose, flavanone-7-0-glucoside-2-0-rhamnosyltransferase, transgenic grapefruit or pomelo

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|---|
| X | BAR-PELED et al. UDP-rhamnose: Flavanone-7-0-glucoside 2" -0-rhamnosyltransferase. The Journal of Biol Chem. 05 November 1991, Vol. 266, No. 31, pages 20953-20959, especially pages 20953 and 20956. | 1 |
| -- | | ---- |
| Y | | 2-7, 13-18, 22-28, 32-33, 62-66, 70-76, 80-83 |
| Y | LEWINSOHN et al. Flavanone Glycoside Biosynthesis in Citrus. Plant Physiol. 1989, Vol. 91, pages 1323-1328, especially pages 1324-1325. | 1-7, 13-18, 22-28, 32-33, 62-66, 70-76, 80-83 |
| Y,P | US 6,054,636 A (FADER, G) 25 April 2000 (25/4/00), see entire document, especially columns 13-16. | 1-7, 13-18, 22-28, 32-33, 62-66, 70-76, 80-83 |



Further documents are listed in the continuation of Box C.



See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be of particular relevance | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *E* earlier document published on or after the international filing date | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *A* document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | |
| *P* document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

15 JUNE 2000

Date of mailing of the international search report

10 JUL 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00038

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|--|
| Y | US 5,859,334 A (BRUGLIERA et al) 12 January 1999 (12/01/99), see entire document, especially columns 8 and 23 | 1-7, 13-18, 22-28, 32-33, 62-66, 70- 76, 80-83 |

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL00/00038

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 8-12, 19-21, 29-31, 67-69, 77-79
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

claims encompass nucleotide sequences and no CRF diskette has been submitted.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-33, 62-83

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL00/00038

A. CLASSIFICATION OF SUBJECT MATTER: US CL :

800/278, 286, 295, 316; 536/23.1, 23.2, 23.6, 24.5; 435/69.1, 419, 468

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-33, 62-83, drawn to an isolated polynucleotide and transgenic plants or plant cells with inhibited expression of flavanone-7-O-glucoside-2"-rhamnosyl-transferase.

Group II, claim(s) 34-42, 46-47, drawn to transgenic plant cells expressing flavanone-7-O-glucoside-2"-rhamnosyl transferase polynucleotide in the sense orientation.

Group II, claim(s) 43-45, 48-61, drawn to a commercial process of producing neohesperidin/dihydrochalcone using transformed microbial cells.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The claimed isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide with a flavanone-7-O-glucoside-2"-O-rhamnosyl-transferase activity is anticipated by Bar-peled et al who teach a purified and characterized flavanone-7-O-glucoside-0-rhamnosyl-transferase for the isolation of the gene which may enable its use in genetic engineering to modify grapefruit bitterness (see, e.g., page 20953 and page 20956, last full paragraph).

The invention of Group I, drawn to an isolated polynucleotide and transgenic plants with inhibited expression of flavanone-7-O-glucoside-2"-rhamnosyl transferase, requires transformation by antisense, overexpression or knock out and regeneration of the transformed cells not required by any of the other group.

The invention of Group II, drawn to transgenic plant cells, requires plant cells and a polynucleotide expressed in the sense orientation for protein production in plants not required by any of the other group.

The invention of Group III, drawn to a commercial process of producing neohesperidin, requires transformed microbial cells, extraction and purification methods and hesperidin and in vitro enzyme reactions, not required by any of the other group.

BAR-PELED et al. UDP-rhamnose:Flavanone-7-O-glucoside-2"-O-rhamnosyltransferase. The Journal of Biological Chemistry. 05 November 1991, Vol. 266, No. 31, pages 20953-20959.

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